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Laboratory Investigations

Efficient Gene Transfer into Normal Human Skeletal Cells Using Recombinant Adenovirus and Conjugated Adenovirus-DNA Complexes

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Abstract. In order to assess efficient DNA gene transfer into human primary cell cultures derived from the skeleton we tested two viral-based procedures. First, replication-deficient recombinant adenoviruses (ADV) were used to infect post-confluent human marrow stromal fibroblasts (HMSF) and human trabecular bone (HTB) cells. Both cell types were readily infected by modified adenoviral vectors carrying a reporter gene making this virus an attractive candidate to facilitate DNA gene transfer. In a second approach we coincubated DNA with ADV that had polylysine (PLL) covalently attached. With this ADV/PLL/DNA complex, very efficient gene transfer into multilayered HMSF and HTB cell cultures was observed, and DNA coincubated with unmodified ADV failed to be effectively transferred. These data imply that the covalently bound PLL more effectively binds exogenous DNA, resulting in a highly efficient internalization event in both cell types. Thus, this latter method has many advantages over conventional ADV gene transfer procedures. It is simple, rapid, and it does not require engineering of DNA into the viral genome, thereby allowing transfer of large fragments of DNA.

Key words: Transfection — Gene transfer — Adenovirus — Polylysine-conjugate — Skeletal cells.

Replication-deficient recombinant adenoviruses (ADV) have been employed to introduce a wide variety of recombinant genes into numerous established cell lines and primary cultures [1]. Adenoviruses bind to cell surface receptors and enter the cells via receptor-mediated endocytosis [2]. Because of their endosomolytic properties, the viruses destroy the endosomal membrane which results in passage of the packed DNA into the cytoplasm [3, 4]. In certain cells, DNA can be passively internalized when bound to the external surface of the viral particles [5]. Efficiency of this process can be increased by coupling ADV to variants of polylysine (PLL) [6, 7]. The essential elements for DNA transfer appear to be a cell-surface receptor-binding site provided by the ADV and a DNA-binding moiety which is provided by PLL [8].

DNA transfer into postconfluent (i.e., nondividing) human skeletal cells presents a major challenge using conventional, nonviral procedures. We explored two adenoviral-based methods in order to find a more efficient way to transfer DNA into mineralized tissue-derived cells. Human marrow stromal fibroblasts (HMSF) derived from bone marrow stroma representing a population of undifferentiated precursors and the more mature human trabecular bone (HTB) cells derived from mineralized trabeculae have been used to study events associated with bone formation [9, 10]. Both cell types multilayer after a certain period in culture and deposit mineral within their extracellular matrix, resulting in the development of bone tissue-like structures.

In this report we show that replication-deficient ADV containing a reporter gene as well as complexes made of PLL-conjugated ADV with DNA can effectively transfect HMSF and HTB cells. Thus, both procedures offer a novel approach to study DNA-mediated events in multilayered primary cultures of human skeletal cells.

Materials and Methods

Primary Cell Cultures

Human bone marrow aspirates and bone specimens were obtained from patients undergoing corrective surgery under an institutionally approved procedure (protocol D-0188). Aspirates were processed as described in Krebsbach et al. [11]. For HMSF in primary culture, cells were plated at 3×10^4 cells per a 100-mm dish. For passaged HMSF strains, cells were plated at a density of 6.7×10^5 nucleated cells per cm^2 and passaged by day 14 when HMSF colonies began to merge. For transfection experiments, passaged HMSF strains were used at passage five. Primary cultures of HTB cells were obtained by cleaning trabecular bone fragments followed by collagenase P treatment. After about 6 weeks the bone cells reached confluency [10].

Replication-defective Adenoviruses

Variants of ADV were used that contained a Rous sarcoma virus

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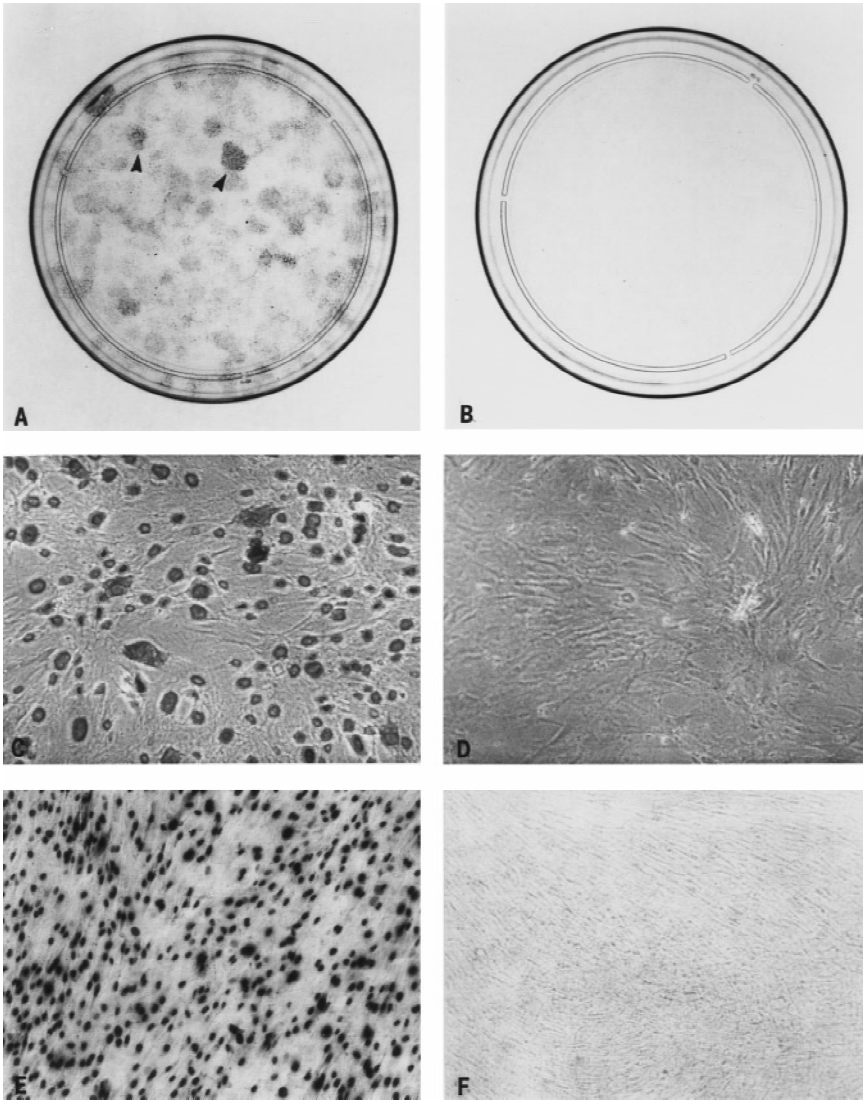

Fig. 1. Primary culture of human marrow stromal fibroblasts infected with replication-deficient adenovirus. Cells plated at a very low density in a 100-mm plate were cultured for 1 week to allow colony formation. After infection with an adenovirus containing an RSV-driven nuclear localization signal for β-galactosidase and incubation for 48 hours, cells were fixed and stained with X-gal for detection of reporter DNA. Arrowheads point out stronger staining in diverse colonies, each presumed to be derived from a single skeletal cell **(A).** The control does not show any staining (panel B). Cells from one of the β-galactosidase-positive colonies in panel A are shown in higher power magnification (**C** and **D**). Almost all nuclei stained for β-galactosidase indicates successful transfection with the adenovirus construct (panel C) whereas in the control, no staining is visible (panel D). Magnification ×200. Primary cells of the fifth passage were cultured for 3 weeks to obtain multilayered cultures. After incubation for 48 hours with the RSV/β-galactosidase containing virus the cells were fixed and stained with X-gal. Transfected cells in **(E)** show very strong staining in their nuclei; the control is free of staining **(F).** Magnification ×100.

(RSV)-promoter controlling the expression of a β-galactosidase gene with a nuclear localization signal. Replication-defective ADV (gift from T. Shenk, Princeton University) for PLL complex formation were amplified as follows [12]. Sixty 150-mm culture plates of subconfluent 293 cells (ATCC #CRL11753) cultured in IMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin were infected with approximately 4×10^8 plaque-forming units (PFU) per plate and the viral infection was allowed to proceed until cytopathic effects were noticed (between 24–48 hours). Infected cells were removed from the plate by vigorous pipetting and concentrated by centrifugation for 10 minutes at 1500 rpm. The cell pellets were combined and resuspended in 7 ml of medium. Cells were lysed by five cycles of freeze thawing, cellular debris was removed by centrifugation at 7000 rpm at 4°C for 7 minutes, and viral particles were purified by twice banding in cesium chloride. After dialysis in 10 mM Tris, pH 7.4, 1 mM $MgCl_2$, and 10% glycerol at 4°C, the quantity of PFU in the preparation was estimated by incubating 293 cells embedded in 1% agarose with serial dilutions of an aliquot of the viral preparation. Evaluation and plaque formation was carried out after 2 weeks of infection. Typically ~2–4 × 10^{11} PFU were obtained from one viral preparation.

Modification of Adenovirus for DNA Complex Formation

Approximately 1.4×10^{11} viral particles were combined with PLL (Sigma, St. Louis, MO; 20.5 kDa) at a concentration of 16 μM, along with 1-ethyl-3(3-dimethylaminoproyl) carbodiimide (EDC) at a final concentration of 2.6 mM in 4 ml. After incubation on ice for 4 hours the untreated components were removed by ultracentrifugation at 35,000 rpm for 18 hours on a cesium chloride gradient (1.35 g/ml). The purified virus was collected as described above, dialyzed against 2 M NaCl, and stored at −20°C in 10% glycerol [13]. The DNA used for the complex formation was routinely purified by a Qiagen Mega-Prep kit (Qiagen, Santa Clarita, CA). The ADV/PLL/DNA complexes were made by combining 10 μg of DNA in 250 μl of HBS (150 mM NaCl, 20 mM HEPES, pH 7.3) with 1×10^{10} modified adenoviral particles in 300 μl of HBS and incubated at room temperature for 30 minutes. An additional 250 μl of HBS was added and the mix was incubated for another 30 minutes [14]. Various doses of the viral complex were added directly to the cells and transgene activity was assayed after 48 hours.

Transfection of HMSF and HTB Cells

For transfection of HMSF, cells at passage 5 were plated at a density of ~2×10^6 cells/100-mm plate and cultured until they reached confluency and became multilayered. For transfection of HTB cells, primary cultures were used at confluency. Fresh me-

dium containing serum was added together with ADV/PLL/DNA complexes at concentrations ranging from 5 to 50 PFU per cell. For ADV/PLL/DNA complex formation, 10 μg of CMV/β-galactosidase reporter DNA was used together with 1×10^{10} particles of ADV conjugated to PLL, and complexes were prepared as described above. The cells were incubated with various amounts of complex solution at 37°C for 48 hours followed by analysis of gene transfer.

Quantitation of Gene Transfer

To determine the efficiency of DNA transfer, HMSF and HTB cells were fixed by brief treatment in PBS (0.02 M sodium phosphate/0.15 M NaCl, pH 7.4) containing 2% formaldehyde and 0.2% glutaraldehyde. Cells were stained for β-galactosidase by incubation in a solution of X-gal (1 mg/ml X-gal, 1 mM $MgCl_2$, 5 μM $K_3Fe(CN)_3$, and 5 μM $K_4Fe(CN)_6 \cdot 3H_2O$ in PBS, pH 7.5). Numerical evaluation of β-galactosidase activity was performed using the Galacto-Light™ system (Tropix, Bedford, MA). Luciferase activity was measured according to a standard assay for luciferase (Promega, Madison, WI). Light emission was measured in a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA).

Results and Discussion

Transfection of Human Marrow Stromal Fibroblasts with Replication-deficient Adenoviruses

Cultures of HMSF colonies were infected for 48 hours with the β-galactosidase containing ADV, fixed and stained with the chromogenic substrate X-gal. All of the HMSF colonies (Fig. 1, panel A) showed staining of their nuclei. However, staining of the different colonies was variably strong (arrowheads) (panel A). High power visualization of a positive colony in Figure 1A shows that all cells stained positive for β-galactosidase (Fig. 1, panel C). In multilayered cultures of passaged HMSF strains infected after 3 weeks, β-galactosidase activity was detectable in many nuclei (panel E). Cells in control experiments without virus added were free of staining (panels B, D, F). Numerical evaluation of β-galactosidase activity in HMSF colonies infected with 5 PFU indicated a 9300-fold increase over background (Fig. 2). Thus, these data show that greater than 90% of the post-confluent HMSF cells showed strong β-galactosidase activity indicating successful gene transfer. The differences in color, i.e., expression of the reporter gene between primary HMSF colonies (Fig. 1), is most likely due to the heterogeneous nature of the HMSF which arises from clones with varying phenotype. Since each HMSF colony derives from a single stromal precursor cell [15] and these cells represent a heterogeneous population [9], HMSF colonies may vary in general aspects including different amounts of ADV receptors, different uptake of DNA during transfection, or as a third possibility, these colonies might have differential RSV-promoter utilization. The presence of the virus had no effect on the expression of several skeletal markers such as alkaline phosphatase, osteonectin, and biglycan (not shown).

Transfection of Human Trabecular Bone Cells with Replication-deficient Adenoviruses

Substantial β-galactosidase gene expression was detectable (using the same protocol as described above) in confluent HTB cells infected with the RSV/β-galactosidase containing virus. Stained nuclei of bone cells surrounding bone

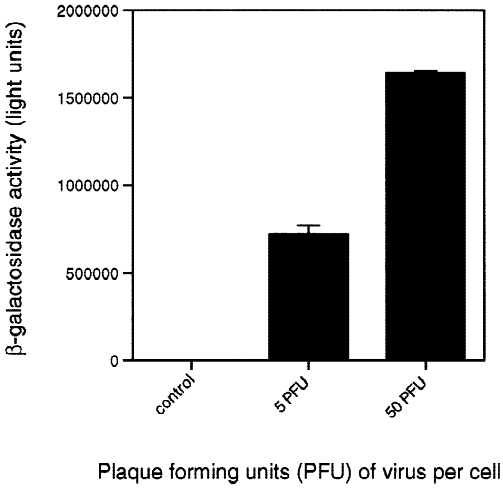

Fig. 2. Quantitation of gene expression in primary human marrow stromal fibroblasts infected with virus. For quantitation, the cells were harvested 48 hours after infection with the RSV/β-galactosidase containing virus and the expressed light units were measured in a luminometer. In transfected cells using five plaque-forming units (PFU) of virus per cell, a 9300-fold increase of β-galactosidase light units over the control was observed.

chips from which the cells emanated are shown in Figure 3 (panel A). However, some heterogeneity in staining was apparent. In a control without virus the cells showed no staining (panel B). Quantitation revealed a 500-fold increase of β-galactosidase activity over background (data not shown). The above experiments indicate that both HMSF and HTB cells were consistently infected by the replication-deficient ADV indicating that the virus can somehow bind to and enter these cells. However, comparison of both cell types revealed that HTB cells had a lower transfection efficiency than HMSF.

Complex Formation of DNA with Polylysine-conjugated Replication-deficient Adenoviruses

Luciferase reporter DNA (pGL3; Promega) was incubated with increasing titers of ADV/PLL and the electrophoretic mobility of the formed ADV/PLL/DNA complexes in a 1% agarose gel was tested (Fig. 4). In lane 2, 10 μg of DNA without viral complexes was used as a control. With increasing amounts of ADV/PLL (25–150 μl), the resulting ADV/PLL/DNA complexes migrated more slowly during electrophoresis. Using either 100 or 150 μl of ADV/PLL, the complexes were unable to penetrate the gel indicating that essentially all of the DNA added was used to form complexes (lane 5 and 6, respectively). We speculate that under these conditions the negatively charged DNA becomes neutralized and the resulting ADV/PLL/DNA complex is unable to migrate in an electric field. Because complexes with least mobility were the most efficient in gene transfer, we used this parameter to estimate the optimal DNA/complex ratio for gene transfer.

DNA Transfer with Polylysine-conjugated Adenoviruses

Adenovirus-assisted endocytosis into HMSF was tested comparing cointernalization of DNA plus unmodified virus

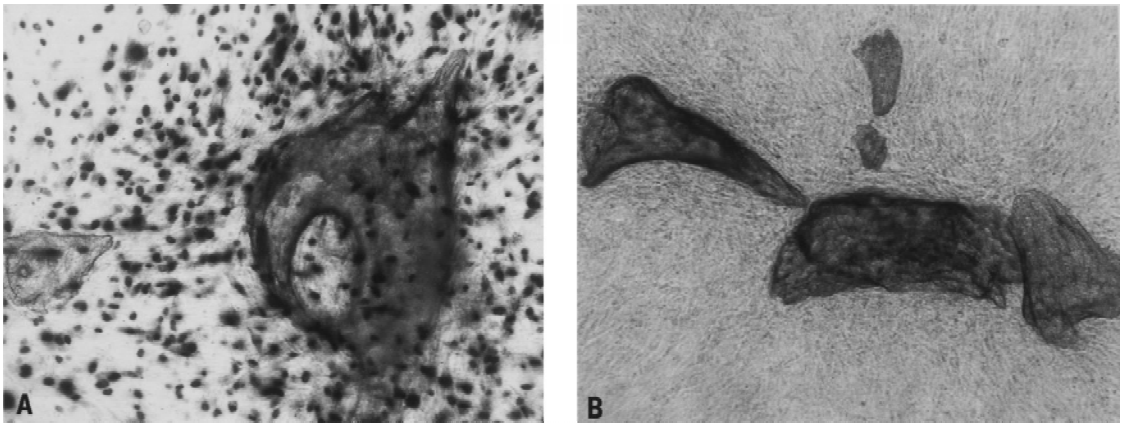

Fig. 3. Human trabecular bone cells infected with RSV/β-galactosidase containing virus. The bone cells were grown over a period of 6 weeks until they became multilayered. Infection with RSV/β-galactosidase containing virusand X-gal staining was performed as described in Figure 1. **(A)** X-gal-positive nuclei of cells surrounding bone fragments are visible. The control shows no staining **(B).** Magnification ×100.

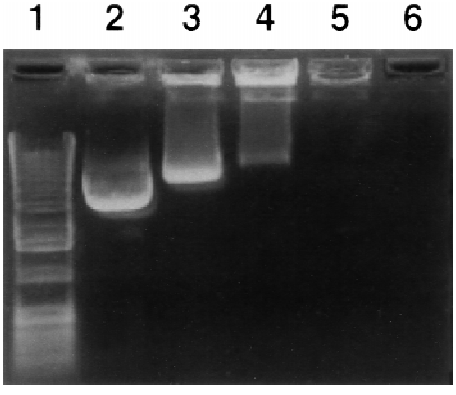

Fig. 4. Characterization of DNA incubated with PLL-conjugated ADV. Luciferase reporter DNA was incubated with different doses of PLL-conjugated ADV and the mobility of the formed ADV/PLL/DNA complexes during electrophoresis in a 1% agarose gel was tested. Lane 1 are the markers. In lane 2, 10 μg of DNA without viral complexes was used as a control. With increasing amounts of ADV/PLL and constant DNA (10 μg), the resulting complexes migrated more slowly (lane 3: 25 μl / lane 4: 50 μl). With 100 and 150 μl of ADV/PLL, essentially all of the DNA added was used to form complexes and these complexes were unable to penetrate the gel (lanes 5 and 6, respectively).

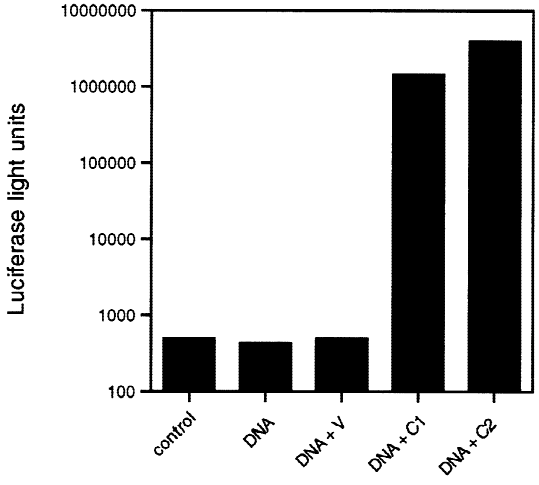

Fig. 5. Quantitation of DNA transfer in human marrow stromal fibroblasts using complexes. Passaged HMSF strains were transfected with equal amounts of pGL3 luciferase reporter DNA (10 μg) and either unmodified virus (DNA + V) or two different doses of preformed ADV/PLL/DNA complexes (DNA + C1:500 μl / DNA + C2:1 ml). In controls, no DNA was added. Standard deviation was less than 1%.

with transfection, using PLL-conjugated ADV/DNA complexes with a DNA reporter construct containing a luciferase gene driven by the SV40-promoter (pGL3). Passaged HMSF strains were transfected either with DNA alone, with DNA plus virus, or with 500 μl and 1 ml of preformed ADV/PLL/DNA complexes. Cells transfected with DNA alone and with DNA plus unmodified virus resulted in essentially no gene transfer (Fig. 5). However, transfection of HMSF with the two different doses of complex resulted in luciferase values that increased in a dose-dependent manner and were significantly higher (3000 and 7500-fold, respectively) than control samples in which no DNA was added.

Different doses of complex (0.1–200 μl), all containing 10^{10} viral particles and equal amounts of CMV/β-galactosidase reporter DNA (10 μg), were added to HTB cells in culture (Fig. 6). As a control, no complexes were added. With higher doses of complex there was a dose-dependent increase in gene transfer after 48 hours of incubation with maximum levels (15-fold over background) achieved with 200 μl of complex per 100-mm plate. The ADV/PLL/DNA complexes were substantially more efficient compared with transfection using ADV/DNA or DNA alone. However, gene transfer into the more mature HTB cells appeared to be less efficient than into the HMSF precursor cell population using PLL-conjugated ADV.

Expression of Transduced Genes

Even though these ADV-based methods offer numerous advantages for gene transfer, they are transient in nature since there is little or no integration of DNA into the host genome. However, viral expression in confluent HMSF cultures tested by Northern blot analysis showed that the transgene

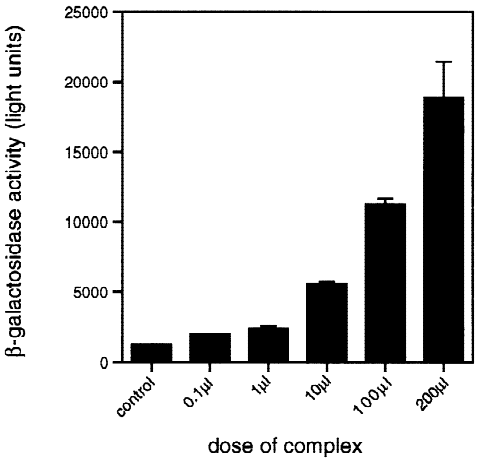

Fig. 6. DNA transfer into human trabecular bone cells using complexes. Multilayered cells were transfected with different doses of ADV/PLL/DNA complexes carrying CMV/β-galactosidase as reporter DNA. For complex formation, 10 μg of DNA and 10^{10} viral particles were used in each lane.

was still present after 28 days in culture (data not shown). Transgene expression after *ex vivo* infection followed by implantation of the cells under the skin of nude mice [15] was analyzed. Even though maximal β-galactosidase activity was observed at 2 weeks and then decreased with time after implantation, it was still detectable at 6 weeks postinfection (14-fold over control cells without viral treatment). We assume that in this experiment cells in implants underwent cell division and that the decrease in activity was due to a ''dilution'' of the transgene. Theoretically this could be explained with the fact that the ADV genome does not incorporate into the host genome. Nevertheless, despite its transient nature, it is clear that both recombinant ADV and PLL-conjugated ADV/DNA complexes are highly successful in transferring genes into primary cultures of human skeletal cells. Therefore we conclude that both methods are greatly suitable for the investigation of DNA-mediated events such as promoter function or signal transduction.

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